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Spectral sensitivity of melatonin synthesis suppression in *Xenopus* eyecups

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Abstract

Melatonin synthesis in retinal photoreceptors is stimulated at night by a circadian oscillator and suppressed acutely by light. To identify photoreceptor mechanisms involved in the acute suppression of melatonin synthesis, an action spectrum was measured for dark-adapted *Xenopus laevis* eyecups at night. Intensity–response curves at six wavelengths from 400 to 650 nm were parallel, suggesting that a single photopigment predominates in melatonin suppression. Half-saturating intensities at 400, 440, 480, and 533 nm were not significantly different from one another, at 1–2 $\times$ 10^8 quanta cm^{-2} s^{-1}. Significantly higher intensities of 580- and 650-nm light were required for melatonin suppression. These results indicate a predominant role for the principal green-absorbing rods in acute regulation of retinal melatonin synthesis in response to light, and argue against an important role for the red-absorbing cones. Higher than expected sensitivity at short wavelengths suggests that photoreceptors sensitive to blue and/or violet light may also contribute to melatonin suppression.

Keywords: Action spectrum, Retina, Photoreceptors, Circadian

Introduction

Melatonin (5-methoxy-N-acetyltryptamine) is synthesized and released by photoreceptor cells in vertebrate retinas (Cahill & Besharse, 1992; Zawilska & Iuvone, 1992; Wiechmann & Craft, 1993; Bernard et al., 1997). The activities and mRNA levels of two enzymes in the melatonin synthetic pathway, arylalkylamine-N-acetyltransferase (AANAT) and tryptophan hydroxylase, are regulated by light and a circadian oscillator that is located within the photoreceptor layer (Besharse & Iuvone, 1983; Thomas & Iuvone, 1991; Cahill & Besharse, 1992, 1993; Green & Besharse, 1994; Bernard et al., 1997). This regulation of the melatonin synthetic pathway results in daily rhythms of melatonin release, with highest levels at night (Cahill & Besharse, 1990, 1991). Retinal melatonin acts as a paracrine signal for dark in regulation of several aspects of retinal physiology, including photoreceptor disk shedding, retinomotor movements, retinal dopamine release, and visual sensitivity (reviewed by Cahill & Besharse, 1995).

Light can affect retinal melatonin synthesis through multiple pathways: Light acutely suppresses the activity of retinal AANAT (Binkley et al., 1979; Iuvone & Besharse, 1983), and daily light–dark cycles have an indirect effect through entrainment of the circadian oscillator. Both acute and circadian responses can result from an action of light directly on photoreceptors (Cahill & Besharse, 1992, 1993). Additionally, light stimulates the release of dopamine from amacrine and interplexiform cells, and dopamine can suppress melatonin synthesis and reset the oscillator (Iuvone & Besharse, 1986; Iuvone et al., 1987; Boatright et al., 1989; Cahill & Besharse, 1991; Witkovsky et al., 1993).

To identify photoreceptor mechanisms involved in acute photic regulation of melatonin synthesis, we measured an action spectrum for suppression of melatonin release from cultured *Xenopus* eyecups. There are at least four different photopigments in *Xenopus* retina. Microspectrophotometric studies have demonstrated that the majority of rods absorb green light (λ_{max} = 522 nm), that another class of rods absorbs blue light (λ_{max} = 445 nm), and that the majority of cones absorb red light (λ_{max} = 612 nm) (Witkovsky et al., 1981*a*). In addition, a *Xenopus* cone opsin that forms a violet-sensitive pigment has been cloned (Starace & Knox, 1997), and two classes of cones, in addition to the predominant red-sensitive class, have been identified by immunocytochemistry (Zhang et al., 1994).

Methods

Male *Xenopus laevis* (length 5–6.5 cm) were obtained from Nasco (Fort Atkinson,WI) and maintained under a cycle of 12-h light and 12-h darkness for at least 2 weeks prior to use. Animals were decapitated and pithed, and the eyes were removed within 2 h of the end of the light period. Eyecups including the retina, choroid, and sclera were prepared by making a circular incision just anterior to the *ora serrata*, then removing the cornea, iris, and lens, as described previously (Besharse & Dunis, 1983).

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Eyecups were cultured individually at room temperature in the wells of two-chamber culture slides (Lab-Tek, Nalge Nunc, Rochester, NY), modified as follows: Teflon-coated stainless-steel wire was coiled to the approximate diameter of the eyecups and a short segment of coil was fixed to the bottom of each well. Eyecups were placed vitreal side up inside these coils, which held the retinas directed upward toward the light source. The sides of the chambers were masked with black tape so that only light from above reached the eyecups. The chambers were placed in a gas-tight lucite incubator on a shaker rotating at 60 rpm. Eyecups were cultured at room temperature (~23°C) in defined culture medium (Cahill & Besharse, 1991) in a humidified atmosphere of 95% O_2/5% CO_2. The serotonin precursor 5-hydroxy-L-tryptophan (5HTrp, 100 μM) was added to the culture medium to enhance melatonin production (Cahill & Besharse, 1990).

To produce monochromatic light, a collimated beam from a 100-W tungsten-halogen lamp was directed through bandpass interference filters with 10 nm nominal half-bandwidths. A mirror reflected the monochromatic light downward onto the culture chambers. The chambers were covered by neutral density filters, so that groups of eyecups were exposed to intensities that differed in approximately 1 log unit steps. The intensity (in W/cm^2/s) of unattenuated monochromatic light at the position of each culture chamber was measured by a calibrated IL700 radiometer with an irradiance detector probe (International Light, Newburyport, MA).

The rhythmic change in melatonin release varies less among eyecups than does the absolute amount of melatonin release (Cahill & Besharse, 1991). Therefore, for each eyecup, melatonin release during exposure to light was normalized relative to melatonin release during a preceding dark period. All eyecups were first cultured in 2 ml medium in darkness for 3.5 h, starting at the normal time of light offset. The culture medium was then collected from each well, and 2 ml fresh medium was added, using infrared illumination. The eyecups were then cultured for another 3.5 h under monochromatic light, darkness, or bright white light (~300–1000 lux), and the culture medium was collected again. The melatonin content of unextracted culture medium samples (100 μl/assay tube) was determined by radioimmunoassay (RIA) using the antiserum developed by Rollag and Niswender (1976); the RIA has been validated for measurement of melatonin in our culture medium (Cahill & Besharse, 1990).

Seven groups of four eyecups each were used to test a single wavelength in each experiment; five groups were each exposed to a different intensity of monochromatic light, one was exposed to darkness to measure maximal melatonin release, and one was exposed to bright white light to measure maximal suppression. The data for each wavelength were normalized relative to the dark control (set to 1.0) and the bright white light control (set to 0.0). The Allfit curve-fitting program (De Lean et al., 1978) was used to simultaneously derive four-parameter logistic equations for the intensity–response data. This program uses a nonlinear, least-squares curve-fitting routine to estimate the maxima, minima, slopes, and the half-saturating intensities of a family of curves, along with confidence intervals for each estimate. To determine whether curves differ in one or more parameters, the curves can be constrained to share selected parameters. If sharing a parameter reduces the goodness of fit, it indicates that the curves differ in that parameter.

Results

During the initial dark incubation, total melatonin accumulation in the medium ranged from 200 to 950 pg/ml. When eyecups were maintained in darkness, melatonin release during the test (second) incubation period was 150–210% of the amount released during the first incubation period, depending on the experiment. The within-experiment coefficient of variation (*CV*) for normalized melatonin release in these dark controls was 11 ± 2% (mean ± S.E.M.). Bright white light during the test incubation suppressed melatonin release to 20–30% of the amount released during the first incubation. The within-experiment *CV* for normalized melatonin release in the bright white light controls was 16 ± 2%.

Suppression of melatonin release by monochromatic light was intensity dependent at all wavelengths tested, and all intensity–response curves could be fit by four-parameter logistic equations. The normalized data and best-fit intensity–response curves derived when all maxima were constrained to 1.0 and all other parameters were free to vary are shown in Fig. 1. When fit independently, the slopes ranged from −0.54 to −0.81. When the curves were constrained to share the same slope, the goodness of fit was not affected, and an overall slope of −0.63 was derived. This indicates that the slopes of the curves are not significantly different, and is consistent with, but does not prove, mediation of the response by a single photopigment. At the highest intensities tested, each of the monochromatic lights suppressed melatonin release to >90% of the maximal suppression observed with bright white light.

An action spectrum for melatonin suppression, derived from the half-saturating intensities of the intensity–response curves, is illustrated in Fig. 2. The half-saturating intensities calculated for 400-, 440-, 480-, and 533-nm light ranged from 1.0×10^8 to 1.8×10^8 quanta/cm^2/s, and were not significantly different from one another. The half-saturating intensities calculated for 580- and 650-nm light were significantly higher (4.3×10^8 and 5×10^9 quanta/cm^2/s, respectively). These data exclude a significant role for the red-absorbing cone in suppression of melatonin production in dark-adapted retina, and indicate a primary role for the principal green-absorbing (522 nm) rod (Fig 2). The sensitivity measured at wavelengths under 500 nm is slightly higher than would be ex-

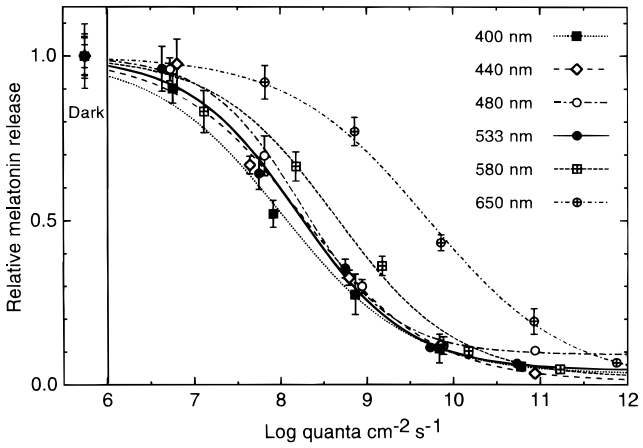

Fig. 1. Intensity–response curves for monochromatic light-induced suppression of melatonin production by cultured *Xenopus* eyecups. Symbols indicate means ± S.E.M. (four eyecups/group). The curves represent four parameter logistic equations fitted to the data by the Allfit program (De Lean et al., 1978). The maxima for all curves were set to 1.0. The fitted slopes, half-saturating intensities and minima of the curves are, respectively: 400 nm, −0.58, 1.0×10^8, 0.03; 440 nm, −0.59, 1.8×10^8, 0.01; 480 nm, −0.80, 1.7×10^8, 0.09; 533 nm, −0.68, 1.6×10^8, 0.04; 580 nm, −0.62, 4.3×10^8, 0.02; and 650 nm, −0.54, 5.0×10^9, 0.05.

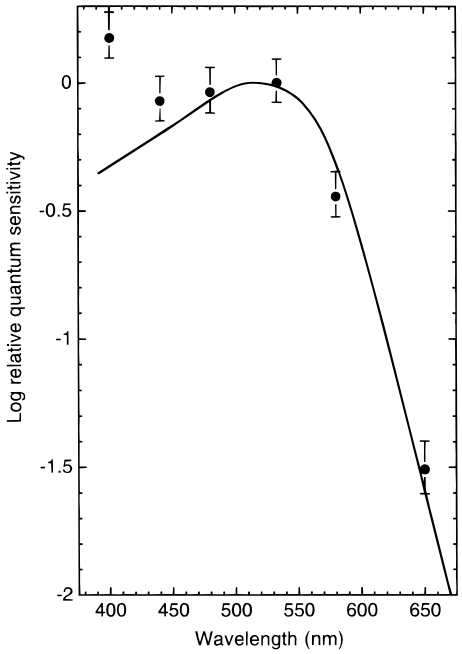

Fig. 2. Action spectrum for suppression of melatonin production by cultured *Xenopus* eyecups. The symbols indicate sensitivities calculated from half-saturating intensities, plotted relative to sensitivity at 533 nm. Error bars indicate approximated relative S.E.M. of the half-saturating intensities. The curve represents the spectral function of the *Xenopus* 522-nm rod, redrawn from Witkovsky et al. (1981*b*).

pected if the green-absorbing rod is the only photoreceptor involved. This may be due to measurement error, or to contributions from the blue-absorbing rod or a UV-sensitive photoreceptor.

Discussion

Our data indicate that green-absorbing (522 nm) rods are the principal photoreceptors involved in acute suppression of melatonin synthesis in *Xenopus laevis* eyecups at night. This conclusion is supported by the finding that the half-saturating light intensity is significantly higher at 580 and 650 nm than at 533 nm. In fact, the sensitivity to 650-nm light is about 1.5 log units lower than the sensitivity to 533-nm light. This sensitivity difference is indistinguishable from the predicted difference for these two wavelengths based on electrophysiological measurement of the threshold of the dark-adapted, aspartate-isolated PIII response in *Xenopus* retina, which is governed by the principal 522-nm rod (Witkovsky et al., 1981*b*). Such data virtually rule out a significant role for the abundant red-absorbing (612 nm) cones in acute suppression of melatonin.

Although the reduced sensitivity at longer wavelengths is consistent with a major role for the principal green-absorbing rods, one would predict that if they were solely responsible for melatonin suppression, the sensitivity would be lower at wavelengths shorter than 533 nm. However, the expected drop in sensitivity at shorter wavelengths is not large (Witkovsky et al., 1981*b*), and there is sufficient variability in our data as to make the short-wavelength measurements statistically indistinguishable from that at 533 nm. The lack of a significant drop in sensitivity at 400 nm suggests that an additional photopigment contributes to the acute suppression of melatonin. Candidates for this effect are the blue-absorbing (445 nm) rods (Witkovsky et al., 1981*a*) and the blue- or violet-absorbing cones (Zhang et al., 1994; Starace & Knox, 1997). Although not directly demonstrated in *Xenopus*, UV-absorbing cones like those observed in another amphibian (Perry & McNaughton, 1991) may be present in *Xenopus* (Zhang et al., 1994). Although these photoreceptor types are present in low numbers compared to the rods and red-absorbing cones, their involvement could counter the expected reduction in sensitivity at wavelengths shorter than 523 nm.

Another feature of our data that is consistent with a major role for rod photoreceptors is that melatonin release from *Xenopus* eyecups is highly sensitive to light. At 533 nm, half-maximal suppression occurs at an irradiance of $\approx$20 quanta/rod/s, assuming a collecting area for each rod of 12 μm^2 (Engbretson & Witkovsky, 1978). It is of some interest to compare the current data with an earlier action spectrum measured for light-evoked cone contraction using similar methodology on eyecups at night (Besharse & Witkovsky, 1992). It was found that green-absorbing rods control light-evoked contraction of red-absorbing cones. As in this study, it was not possible to rule out a minor role for photoreceptors that absorb shorter wavelengths. Although contraction of red-absorbing cones was about 1.5 log units less sensitive to light at 650 nm than at 533 nm (as we found for melatonin suppression), the absolute sensitivity for light-evoked cone contraction at 533 nm was about 20-fold lower (half-maximal contraction at $\approx$384 quanta/rod/s) than for melatonin suppression.

The difference in absolute sensitivity of melatonin suppression compared to cone contraction may be related to different roles for retinal dopamine in the two processes. There is good evidence that light-evoked cone contraction is mediated by dopamine (Pierce & Besharse, 1985; Besharse, 1992), acting *via* D2-like dopamine receptors on the cone cells (Muresan & Besharse, 1993). In particular, light stimulates dopamine release in *Xenopus* eyecups (Boatright et al., 1989; Witkovsky et al., 1993), and D2 receptor antagonists block light-induced cone contraction (Pierce & Besharse, 1985). In other species, light can directly cause cone contraction through an endogenous photoreceptor mechanism (Dearry & Burnside, 1986), but under dark-adapted conditions at night, cones in *Xenopus* eyecups are more sensitive to the dopamine-mediated pathway. In contrast, light-induced suppression of melatonin synthesis appears to be more sensitive to a dopamine-independent pathway. Although application of exogenous dopamine can suppress melatonin release, the acute suppression of melatonin by subsaturating light is not affected by dopamine antagonists (Cahill & Besharse, 1991), unless eyecups are treated with a phosphodiesterase inhibitor (Iuvone et al., 1987). A simple explanation consistent with these data is that the phototransduction pathway that suppresses melatonin is activated at light intensities that are below threshold for evoking dopamine release. At present, however, the absolute sensitivity of light-evoked dopamine release in this system is not known.

The culture conditions used in this study might have influenced the sensitivity of the system by altering neural circuits that contribute to light responses. In particular, a melatonin precursor, 5-hydroxytryptophan, was added to the culture medium to increase melatonin production above normal levels, which are barely detectable by RIA. This treatment bypasses tryptophan hydroxylase, which is normally rate-limiting in melatonin synthesis, and results in elevated melatonin release that reflects the circadian and light-dependent regulation of AANAT (Cahill & Besharse, 1990, 1991). Elevated melatonin is likely to suppress endogenous dopamine

release (Boatright et al., 1994), and may influence responsiveness of horizontal cells (Wiechmann et al., 1988). However, there is no data bearing on whether melatonin affects phototransduction mechanisms within rods or cones.

Most, if not all, of the melatonin synthesized in retina is made by photoreceptors (Cahill & Besharse, 1992; Wiechmann & Craft, 1993). Both rods and cones in *Xenopus* retina have been shown to contain mRNA for tryptophan hydroxylase (Green et al., 1995), suggesting that both cell types may be capable of synthesizing melatonin. However, there is no evidence yet regarding the relative contributions of rods and cones to total retinal melatonin production. The insensitivity of melatonin release to red light is consistent with either a lack of significant melatonin synthesis in red-absorbing cones or regulation of melatonin production in those cones by a rod-initiated pathway.

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